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Protein kinase C protects from DNA damage-induced necrotic cell death by inhibiting poly(ADP-ribose) polymerase-1

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Abstract **The goal of the current study, conducted in freshly isolated thymocytes was (1) to investigate the possibility that the activation of poly(ADP-ribose) polymerase-1 (PARP-1) in an intact cell can be regulated by protein kinase C (PKC) mediated phosphorylation and (2) to examine the consequence of this regulatory mechanism in the context of cell death induced by the genotoxic agent. In cells stimulated by the PKC activating phorbol esters, DNA breakage was unaffected, PARP-1 was phosphorylated, 1-methyl-3-nitro-1-nitrosoguanidine-induced PARP activation and cell necrosis were suppressed, with all these effects attenuated by the PKC inhibitors GF109203X or Gö6976. Inhibition of cellular PARP activity by PKC-mediated phosphorylation may provide a plausible mechanism for the previously observed cytoprotective effects of PKC activators.**

Keywords: Poly(ADP-ribose) polymerase-1; Protein kinase C; Cytotoxicity; Necrosis; Apoptosis; 1-Methyl-3-nitro-1-nitrosoguanidine

1. Introduction

The nuclear enzyme poly(ADP-ribose) polymerase 1 (PARP-1), a highly conserved constitutively-expressed 116 kDa protein is the most abundant isoform of the PARP enzyme family with roles in regulating multiple cellular functions in health and disease (overviewed in [1,2]). Poly(ADP-ribosyl)ation has been implicated in the regulation of multiple physiological cellular functions such as DNA repair, gene transcription, cell cycle progression, cell death, chromatin function and genomic stability [3]. Because PARP-1 becomes activated in response to DNA breaks, the nature of the various endogenous species capable of inducing DNA strand breaks (and thereby activating PARP-1) in various disease conditions became of crucial interest. In addition to hydroxyl radical, peroxynitrite (a reactive nitrogen species formed from the diffusion-limited reaction of nitric oxide and superoxide anion) has been identified as a pathophysiologically relevant trigger of PARP activation [2].

From a pathophysiological standpoint, PARP activation can contribute to the development of disease via two main mechanisms: (a) by driving the cell into an energetic deficit and a state of dysfunction and (b) by catalyzing the activation of pro-inflammatory pathways. As far as the former pathway: PARP-1 functions as a DNA damage sensor and signaling molecule binding to both single- and double-stranded DNA breaks. Upon binding to damaged DNA, PARP-1 forms homodimers and catalyzes the cleavage of NAD^+ into nicotinamide and ADP-ribose to form long branches of $(ADP\text{-}ribose)_n$ polymers on target proteins including histones and PARP-1 itself. This process results in cellular energetic depletion, mitochondrial dysfunction and ultimately necrosis [1,2]. As far as the latter pathway: numerous transcription factors, DNA replication factors and signaling molecules have also been shown to become poly(ADP-ribosyl)ated by PARP-1, but a PARP-mediated activation of the pluripotent transcription factor nuclear factor kappa B (NF-κB) appears to be of crucial importance [1,4]. Multiple lines of studies demonstrate that neutralization of peroxynitrite and/or pharmacological inhibition or genetic inactivation of PARP-1 is therapeutically effective in a wide range of cardiovascular, inflammatory, vascular and neurodegenerative diseases, both by protecting against cell death as well as by down-regulating multiple inflammatory pathways [1].

The activity of PARP-1 at the cellular level is primarily regulated by DNA single strand breaks. These breaks are recognized by zinc-fingers of PARP-1, and induce a conformational change in the enzyme, which, in turn, results in an increased catalysis of NAD^+ to ADP-ribose units and nicotinamide, as the byproduct of the reaction [2]. However, a number of studies have also suggested alternative (i.e. non-DNA break dependent) intracellular regulatory mechanisms for

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Abbreviations: DMSO, dimethylsulfoxide; DTT, dithiothreitol; MNNG, 1-methyl-3-nitro-1-nitrosoguanidine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C

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PARP activity, with consequences for PARP-dependent cellular functional alterations. Some of these regulators (overviewed in [5]) include calcium, female sex hormones, xanthines and other factors. There are two published studies [6,7] demonstrating the ability of protein kinase C (PKC) to phosphorylate PARP-1 in in vitro systems. The current study, conducted in freshly isolated thymocytes has investigated the possibility that the activation of PARP in an intact cell can be regulated by phosphorylation via PKC and examined the consequence of this regulatory mechanism in the context of cell death induced by nitrosative stress.

2. Materials and methods

2.1. Materials

Propidium iodide (PI) was purchased from Molecular Probes (Eugene, OR, USA). All other chemicals, including 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) were from Sigma–Aldrich (St. Louis, MO, USA). The water soluble PARP inhibitor, PJ-34 [8] was produced by Inotek Pharmaceuticals (Beverly, MA, USA).

2.2. Cytotoxicity assay

Thymocytes were prepared according to Bai et al.[9] and Virag et al. [10] MNNG-induced cytotoxicity was measured by propidium iodide (PI) uptake as described previously [9]. Cytotoxicity has also been determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described [11] with the exception that treatments were carried out in Eppendorf tubes and cells were spun down before removal of the medium and addition of dimethylsulfoxide (DMSO).

2.3. PARP activity assay

PARP activity of cells was determined with the traditional PARP activity assay based on the incorporation of isotope from ^{3}H-NAD^{+} into TCA (trichloroacetic acid)-precipitable proteins as described [10].

2.4. Caspase activity assay

Caspase-3 like activity was detected as described previously [12].

2.5. Single cell gel electrophoresis (comet-assay)

Single-stranded DNA strand breaks were assayed by single cell gel electrophoresis (comet assay) according to Singh et al. [13] with modifications as described in [12].

2.6. Immunoprecipitation

PARP-1 phosphorylation was detected by immunoprecipitation. Cells were lysed with sample buffer (150 mM NaCl, 1% Triton-X 100, 50 mMTris–HCl (pH 8.0), 1 mM EDTA, protease inhibitor cocktail (100×), 1 mM NaF, 1 mM Na_2VO_3), sonicated for 20 s. Samples were precleared with 20 μl 50% sepharose-protein-A slurry for 1 h. Samples were incubated for 1.5 h with anti-PARP antibody (4 μg protein/500 μl sample). Fifty microliters of 50% sepharose-protein-A slurry were added and incubated for 1 h. Sepharose-protein-A microbeads were washed three times with sample buffer. Microbeads were mixed with SDS sample buffer then subjected to SDS–polyacrylamide gel electrophoresis in 8% gels and transferred onto nitrocellulose membranes in 25 mM Tris/HCl, pH 8.3, containing 192 mM glycine, 0.02% (w/v) SDS and 20% (v/v) methanol at 250 mA for 90 min.

2.7. Western blotting and immunofluorescence

For Western blotting, cells were lysed with RIPA buffer, sonicated for 20 s, and mixed with SDS sample buffer than subjected to SDS–polyacrylamide gel electrophoresis in 8% gels and transferred onto nitrocellulose membranes in 25 mM Tris/HCl, pH 8.3, containing 192 mM glycine, 0.02% (w/v) SDS and 20% (v/v) methanol at 250 mA for 90 min. immunostaining was performed using polyclonal anti-poly(ADP-ribose), anti-PARP-1 antibody, isoform specific anti-PKC and anti-phosphoserine antibodies according to standard procedures as described in [12,14]. The same anti-PKC antibodies have been used for immunofluorescence stainings with FITC-conjugated secondary antibody according to standard procedures. Nuclei were counterstained with DAPI. Images were acquired with a Zeiss LSM 510 META confocal laser scanning microscope and z-stacked green and blue images were overlaid. Representative images are shown.

2.8. In vitro phosphorylation

Purified PARP-1 enzyme was phosphorylated by purified cPKC mixture (alpha, beta, gamma isoforms) in HEPES assay buffer (200 mM HEPES pH 7.5; 100 mM $MgCl_2$, 10 mM dithiothreitol (DTT)). PKC was diluted in PKC storage buffer (20 mM HEPES pH7,5, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 25% glycerine, 0.02 % NaN_3, 0.05% Triton X-100) to a final concentration of 0.1 μg/ml and was activated by the addition of 0.65 mM $CaCl_2$ and phosphatidyl serine–diolein micellas. ATP mixture (5 μl) containing 0.988 mM ATP and 20-fold diluted ^{32}P-ATP were added to the samples. Samples were incubated for 20, 50 and 90 min. Controls were prepared with the omission of PKC. Samples were mixed with SDS sample buffer and subjected to SDS–polyacrylamide gel electrophoresis in 8% gels. Gels were dried. ^{32}P signals were detected by autoradiography.

2.9. Statistical analysis

All experiments were preformed three times on different days. Student's *t*-test was applied for statistical analysis and for the determination of significance with $P < 0.05$ considered as significant. For the statistical analysis of the comet assay experiments, Mann and Whitney's *U*-test was applied.

3. Results

First, we have tested whether modulation of PKC activity affects PARP activation in thymocytes. Cells were treated with the prototypical DNA-damaging agent MNNG, which has the well-documented effect of inducing DNA single strand breakage, and subsequent PARP activation [12]. As expected, MNNG induced a marked degree of PARP activation (Fig. 1), which was suppressed by the PARP inhibitor PJ34 (Fig. 1). When cells were pretreated for 30 min with the PKC activator phorbol ester phorbol 12-myristate 13-acetate (PMA), a significant suppression of MNNG-induced PARP activation was observed (Fig. 1). The PARP-inhibitory effect of PMA was largely reversed by the compound bisindolylmaleimide (GF109203X), a broad spectrum PKC inhibitor and was partially attenuated by Gö6976 an inhibitor of conventional (calcium-dependent) PKC isoforms (Fig. 1).

In order to investigate the consequences of PKC inhibition in the context of MNNG-induced cell death, we have measured the MNNG-induced changes in overall cell viability, necrotic cell death (propidium iodide uptake) and caspase activation, a measure of apoptotic cell death. As expected, MNNG significantly reduced cell viability and increased necrotic cell death as compared to control (Fig. 2). The cell death was dependent on PARP activation in this model, as evidenced by the inhibitory effect of the PARP inhibitor PJ34 (Fig. 2). Consistent with its PARP-inhibitory effect, PMA pretreatment attenuated MNNG-induced thymocyte cell necrosis (Fig. 2A and B). As shown in previous experiments using PARP-dependent death of murine thymocytes, inhibition of cell necrosis by PARP inhibitors maintains cellular NAD^{+} and ATP levels and increases cellular energy charge [10,15]. These effects are associated with an increase in the population of normal cells (not necrotic or apoptotic), with a decrease in the population of necrotic cells, as well as an increase in the population of apoptotic cells [10,15]. The current results, showing an increase in